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## (57) Abstract

A SOK polypeptide, an isolated DNA having a nucleotide sequence encoding a SOK polypeptide, and a method of determining whether a candidate compound modulates SOK-1 activity or expression, comprising the steps of providing a first and a second recombinant cell expressing a SOK gene; introducing a candidate compound into the first cell, but not into the second cell; measuring a SOK function in the first and second cells; and comparing the results obtained with the first and second SOK transformed cells, wherein an increase or decrease in the SOK function in the first cell compared to the second cell is an indication that the candidate compound modulates SOK expression or activity.

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- 1 -

SOK-1 AND METHODS OF USEStatement as to Federally Sponsored Research

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Background of the Invention

The invention relates to protein kinases and methods of activating or inhibiting the expression of protein kinases.

Mitogen-activated protein kinase (MAPK) cascades have been remarkably conserved in evolution. The core of these cascades is a three-tiered module of serine/threonine kinases that consists of a MAPK-extracellular signal regulated kinase kinase (a MEKK), a MEK, and a MAPK or extracellular signal regulated kinase (ERK). In simple eukaryotes, such as the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*), and the fission yeast, *Saccharomyces pombe* (*S. pombe*), these cascades are activated predominantly by cellular stresses such as nutritional starvation and osmolar stress (reviewed in Elion, *TIBS* 5:322 (1995); Herskowitz, *Cell* 80:187 (1995); and Levin et al., *Cell Biol.* 7:197 (1995)). In mammals, these cascades have evolved to allow responses to complex stimuli (e.g., growth factors and inflammatory cytokines), but in many cases, such as the response to osmolar challenge (Galcheva-Gargova et al., *Science* 265:806 (1994); Han et al., *Science* 265:808 (1994)), the primitive stress responses remain intact.

Epistasis analyses in yeast suggest that the Sterile 20 (Ste20) protein serine/threonine kinases and related protein kinases act upstream of the three tiered module. Three mammalian homologs of Ste20 have been reported to date: p21-activated protein kinase (PAK1) and

- 2 -

related PAKs (Manser et al., *Nature* 367:40 (1994); Martin et al., *EMBO J.* 14:1970 (1995)); germinal center (GC) kinase (Katz et al., *J. Biol. Chem.* 269:16802 (1994)); and mammalian Ste20-like kinase 1 (MST1) (Creasy et al., *J. Biol. Chem.* 270:21695 (1995)). Mammalian Ste20s may function upstream of MEKK/MEK/MAP kinase pathways. PAK1 (Manser et al., *Nature* 367: 40 (1994)) and GC kinase (Katz et al., *J. Biol. Chem.* 269:16802 (1994)) have been shown to be capable of activating mammalian MAPK kinases (Polverino et al., *J. Biol. Chem.* 270:26067 (1995); Pombo et al., *Nature* 377:750 (1995); Zhang et al., *J. Biol. Chem.* 270:12665 (1995)), further illustrating remarkable evolutionary conservation of the MAPK kinases. When co-transfected with MAP kinase, both PAK1 and GC kinase activate the stress-activated protein kinase (SAPK)/c-Jun amino terminal kinase (JNK) cascade. PAK1 also activates the stress activated MAPK, p38, as well.

Ste20 protein kinases can be divided into two families based on their structure and regulation. The first family is the Ste20 family, which includes Ste20, PAK1 and related PAKs. These proteins contain a carboxy terminal catalytic domain and an amino terminal regulatory domain which has a p21<sup>cdc42/rac1</sup> binding region (Manser et al., *Nature* 367:40 (1994); Martin et al., *EMBO J.* 14:1970 (1995)). PAK1 appears to be activated by binding to cdc42Hs or Rac1. Following binding to the small GTP-binding proteins, the kinase undergoes autophosphorylation and is activated. Physiologic activators of PAK1 have been identified, and include the chemoattractant peptide fMetLeuPhe, and Interleukin 1 (IL-1) (Zhang et al., *J. Biol. Chem.* 270:12665 (1995)).

The second family of Ste20s is the Sps1 family. Members of this group include Sps1, which is encoded by the *S. cerevisiae* Sporulation specific 1 gene, which is necessary for spore formation in response to nutritional

- 3 -

starvation; and the mammalian genes MST1 and GC kinase. The catalytic domain is amino terminal in these proteins, and the function of their carboxy terminal regions has not previously been known. These kinases do not contain an identifiable Rac/cdc42Hs binding domain in their non-catalytic regions. The regulation of this family of Ste20s is not well characterized. MST1 appears to be activated by dephosphorylation. Sps1 and its MAPK, Smk1 (Krisak et al., *Genes & Development* 8:2151 (1994)), are transcriptionally regulated, being expressed only at certain stages of the sporulation process, but it is not known if there are other modes of regulation of Sps1. Physiological activators of the Sps1 family of Ste20s have not been previously identified.

#### 15                    Summary of the Invention

The invention is based on the discovery of a novel mammalian protein kinase, SOK-1, that belongs to the Sps1 family of Ste20 homologs. SOK-1 (Ste20 oxidant stress response kinase 1) is a protein kinase which is activated by oxidant stress (e.g., 0.5mM H<sub>2</sub>O<sub>2</sub>).

Accordingly, the invention features an isolated nucleic acid encoding a SOK polypeptide, particularly SOK-1. The naturally occurring SOK polypeptide can be from a mammal, such as a human, non human primate, e.g., baboons, monkeys and chimpanzees, goats, pigs, micro-pigs, guinea pigs, rabbits, rats and mice. This nucleic acid encodes an amino acid sequence with at least 50% (preferably at least 60%, more preferably at least 70%, more preferably at least 85%) identity to the amino acid sequence set forth in Fig. 1 (SEQ ID NO:2). The invention also features a substantially pure preparation of a SOK polypeptide. The SDK polypeptide preferably has an amino acid sequence with at least 50% sequence identity to the sequence set forth as SEQ ID NO:1.

- 4 -

Preferably, the sequence has at least 60%, 70% or 85% sequence identity to the sequence set forth as SEQ ID NO:1. By "SOK polypeptide" is meant all or part of a novel protein kinase, expression of which is activated by oxidant stress.

By "isolated" is meant that the DNA is free of the coding sequences of those genes that, in the naturally-occurring genome of the organism (if any) from which the DNA of the invention is derived, immediately flank the gene encoding the DNA of the invention. The isolated DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a naturally-occurring DNA sequence, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides. The DNAs of the invention therefore include, e.g., a recombinant nucleic acid incorporated into a vector, such as an autonomously replicating plasmid or virus; a cDNA or genomic DNA fragment produced by polymerase chain reaction (PCR) or restriction endonuclease treatment; and recombinant DNA which is part of a hybrid gene encoding additional polypeptide sequences.

Also included in the isolated DNAs of the invention are single-stranded DNAs which are generally at least 10 nucleotides long, preferably at least 18 nucleotides long, more preferably at least 30 nucleotides long, and ranging up to full length of the DNAs encoding a SOK polypeptide.

The single stranded DNAs can also be complementary to a SOK coding strand, so that they can be labelled and used as hybridization probes. Preferably the isolated DNA or its complement hybridizes under stringent conditions to all or part of the nucleotide sequence shown in Fig. 1(SEQ ID NO:2). "Stringent conditions"

- 5 -

include, for example, hybridization at 68° C in 5x SSC/5x Denhardt's solution/1.0% SDS, or in 0.5 M NaHPO<sub>4</sub> (pH 7.2)/1 mM EDTA/7% SDS, or in 50% formamide/0.25 M NaHPO<sub>4</sub> (pH 7.2)/0.25 M NaCl/1 mM EDTA/7% SDS; and washing in 5 0.2x SSC/0.1% SDS at room temperature or at 42°C, or in 0.1 x SSC/0.1% SDS at 68°C, or in 40 mM NaHPO<sub>4</sub> (pH 7.2)/1 mM EDTA/5% SDS at 50°C, or in 40 mM NaHPO<sub>4</sub> (pH 7.2) 1 mM EDTA/1% SDS at 50°C. Moderately stringent conditions including washing in 3 x SSC at 42°C. The 10 parameters of salt concentration and temperature can be varied to achieve the desired level of identity between the probe and the target DNA. For guidance regarding such conditions see, e.g., Sambrook et al., Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Press, 15 Cold Spring Harbor, New York; and Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, 1995.

DNAs of the invention can be incorporated into a vector, which may be provided as a purified preparation. DNA, either by itself, or incorporated into a vector, can 20 be incorporated into a cell, and the cell can be propagated to form an essentially homogenous population of cells (e.g., prokaryotic cells, or eukaryotic cells such as mammalian cells) containing SOK, by methods that are well known to those skilled in the art. An 25 "essentially homogenous" population of cells is one in which at least 99% of the cells contain the vector or the isolated DNA of the invention.

A further aspect of the invention is a method of determining whether a candidate compound modulates the 30 expression or activity of SOK. The method includes the steps of:

- a) providing a first and a second recombinant cell expressing a SOK gene;
- b) introducing a candidate compound into the 35 first recombinant cell, but not into the second cell;

- 6 -

c) measuring a SOK function in the first and second cells;

d) comparing the results obtained with the first and second SOK-transformed cells, wherein an increase or decrease in the SOK function in the first cell compared to the second cell is an indication that the candidate compound modulates SOK expression or activity. In one embodiment of this method, the SOK function to be measured is activation of the gene encoding the transcription factor NF $\kappa$ B. In another embodiment, the function to be measured is protein kinase activity. In other embodiments, the function is arrest of the cell cycle or activation of SOK by H<sub>2</sub>O<sub>2</sub>.

The invention also features a therapeutic composition that includes a SOK polypeptide or DNA as an active ingredient. Such therapeutic compositions can be formulated with a pharmaceutically acceptable carrier. In another aspect, the invention is a method of administering a therapeutically effective amount of a composition of a SOK polypeptide or DNA, or a fragment thereof, to a mammal, to treat a condition characterized by a proliferative response, e.g., to treat a vessel that has sustained balloon angioplasty-induced injury. A "therapeutically effective" amount is an amount that produces a medically desirable result in a patient.

A method of producing a SOK polypeptide is also included in the invention. In this method, cells containing an isolated DNA encoding a SOK polypeptide are cultured under conditions permitting the expression of the SOK polypeptide, and the SOK polypeptide is isolated. Also included in the invention are therapeutic compositions that include DNAs encoding a SOK polypeptide.

In another aspect, the invention is a substantially pure antibody which specifically binds SOK.



- 7 -

An antibody that "specifically binds" to SOK binds to SOK and does not substantially recognize and bind to other antigenically-unrelated molecules. Antibodies according to the invention can be prepared by a variety of methods. 5 For example, a SOK protein or antigenic fragment thereof can be administered to an animal in order to induce the production of polyclonal antibodies. Alternatively, the antibodies can be monoclonal antibodies. Such monoclonal antibodies can be prepared using hybridoma technology 10 (see, e.g., Kohler et al., *Nature* 256:495 (1975); Kohler et al., *Eur. J. Immunol.* 6:292 (1976); Kohler et al., *Eur. J. Immunol.* 6:511 (1976); Hammerling et al., *In Monoclonal Antibodies and T Cell Hybridomas*, Elsevier, New York, 1981).

15 As used herein, "substantially pure" describes a molecule, e.g., a protein, that is substantially free from the components that naturally accompany it. Typically, a compound is substantially pure when at least 60%, more preferably at least 75%, more preferably at 20 least 90%, and most preferably at least 99%, of the total material in a sample is the molecule of interest.

Individuals skilled in the art will recognize that the compositions of the invention can be assembled in a kit for the detection of SOK polypeptides or RNA. 25 Typically, such kits include reagents containing the DNAs or antibodies of the invention with instructions and suitable packaging for their use as part of an assay for SOK. For example, a kit can contain an anti-SOK antibody that is capable of specifically forming an immunocomplex 30 with SOK in a sample, a solid support to which the antibody is bound, and means to detect the immunocomplex.

In another aspect, the invention features a kinase inactive mutant of a SOK polypeptide, or a DNA encoding such a mutant. By "kinase inactive mutant" is meant a

- 8 -

SOK polypeptide which has been altered so that the kinase domain is less active than in the wild-type SOK. Such mutants preferably show 50% or less of the kinase activity of wild-type SOK; more preferably, 25% or less; 5 more preferably, 10% or less; and most preferably, 5% or less of the kinase activity of wild-type SOK. One embodiment is a kinase inactive mutant of SOK-1, in which the invariant lysine in the ATP binding site has been substituted with an arginine.

10       The invention also features a therapeutic composition containing a kinase inactive mutant of a SOK polypeptide, or DNA encoding such a mutant, as an active ingredient. In another aspect, the invention features a method of downregulating the gene encoding NF $\kappa$ B by  
15 administering a therapeutically effective amount of a kinase inactive mutant of a SOK polypeptide, or a DNA encoding such a kinase inactive mutant.

Biologically active fragments of SOK polypeptides, and DNAs encoding such polypeptides, are also included  
20 in the invention. An example of such an active fragment is the portion of the SOK-1 polypeptide corresponding to the noncatalytic carboxy terminal region of SOK-1. A "biologically active" fragment is a fragment having at least 10% of the activity of SOK in specific functions,  
25 e.g., induction of cell cycle arrest. For example, a biologically active fragment can have 30%, 50%, 80%, 90% or up to 100% or more of the activity of SOK. Such fragments include that encoded by amino acids 286 to 426 of SOK-1, and that encoded by amino acids 286 to 336 of  
30 SOK-1. Therapeutic compositions of the invention include such active fragments, or DNAs encoding such fragments, formulated with a pharmaceutically acceptable carrier. A therapeutically effective amount of such a composition is administered to a patient, e.g., to treat  
35 a condition characterized by a proliferative response,

- 9 -

such as balloon angioplasty-induced injury, inflammatory responses, or cancer.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. All publications mentioned herein are incorporated by reference. The examples which follow are illustrative only, and not intended to be limiting.

Other advantages and features of the invention will be apparent from the detailed description, and from the claims.

#### Brief Description of the Drawings

Fig. 1 is a diagram of the nucleotide and predicted amino acid sequences of human SOK-1.

Fig. 2 is a diagram showing an alignment of the amino acid sequence of the catalytic domain of SOK-1 with the amino acid sequence of the catalytic domains of other Ste20 homologs.

Fig. 3 is an autoradiogram of a Northern blot of RNA from various tissues, hybridized to a SOK-1 probe.

Fig. 4A is an autoradiogram of a Western blot of cells transfected with a HA epitope tagged-SOK-1 gene, probed with anti-HA antibody.

Fig. 4B is an autoradiogram of cells transfected with a HA epitope-tagged SOK-1 and immunoprecipitated with anti-HA antibody, followed by immune complex kinase assay using MBP as substrate.

Figs. 5A-F are immunofluorescent stains of SOK-1-transfected cells, showing the subcellular localization of SOK-1.

Fig. 6 is a diagram showing the effect of Protein Phosphatase 2A (PP2A) and autophosphorylation on SOK activity.

- 10 -

Fig. 7 is a diagram showing the effect of the C-terminal non-catalytic region on SOK-1 kinase activity.

Fig. 8 is a diagram showing the kinetics of activation of SOK-1 by  $H_2O_2$ .

5 Fig. 9A is a diagram showing the effect of SOK-1 on the p38 cascade.

Fig. 9B is a diagram showing the effect of SOK-1 on the ERK1 cascade.

#### Detailed Description

10 Mammalian homologs of the yeast serine/threonine protein kinase, Ste20, can be divided into two groups based on their regulation and structure. The first group, the Ste20 family, includes PAK1 and is regulated by Rac1 and cdc42Hs. Activators of protein kinases in  
15 the Ste20 family have been identified. In contrast, little has been known about activators, regulatory mechanisms or physiological roles of the second family, the Sps1 family, which includes GC kinase and MST1. The present invention is based on the identification, cloning  
20 and characterization of a human Ste20 homolog, SOK-1. Like members of the Sps1 family of Ste20 homologs, SOK-1 is characterized by an amino terminal catalytic domain. SOK-1 is positively regulated by phosphorylation, and is negatively regulated by its noncatalytic carboxy terminal  
25 region. There is no significant sequence similarity between this noncatalytic regulatory region and any other protein kinases.

SOK-1 is markedly activated by depletion of intracellular ATP stores, an important component of  
30 ischemia. This novel protein kinase is also activated by oxidant stress, and is the first mammalian Ste20 known to be activated by any cellular stress. This novel protein kinase is not activated by growth factors, alkylating agents, cytokines or environmental stresses including

- 11 -

heat shock and osmolar stress. SOK-1 does not act as part of a generalized stress response pathway, but is activated relatively specifically by oxidant stress. Oxidant stress is a prominent component of ischemia and of reperfusion of ischemic tissue.

SOK-1 activates the transcription factor NF $\kappa$ B, which is implicated in a host of pathological conditions including inflammation and autoimmune syndromes. A kinase inactive mutant of SOK-1 can inhibit NF $\kappa$ B activity. SOK-1 also induces cell cycle arrest via a pathway that is independent of other stress activated protein kinases known to effect cell cycle arrest. This cell cycle arrest is mediated by the noncatalytic subunit of SOK-1.

Unlike GC kinase, a member of the Ste20 family, and PAK1, a member of the Sps1 family, SOK-1 does not activate any of the known MAPK pathways, such as SAPK/JNK, p38 or ERK1/-2. SOK-1 thus defines a novel stress response pathway which is likely to include a unique stress-activated MAP kinase cascade. The data suggest that SOK-1 functions similarly to yeast Ste20s, which transduce signals in response to environmental stress.

## Materials and Methods

### Isolation and analysis of SOK-1 cDNA

#### Degenerate sense

[GA(A/G)(C/T)TIATGGCIGTIAA(A/G)CA] and antisense [TTIGCICC(T/C)TTIAT(A/G)TCIC(G/T)(A/G)TG] primers were used to amplify DNA from a human placenta cDNA library using Taq polymerase. The PCR products were ligated into the pCRII vector (Invitrogen). A 350 bp fragment was obtained which was not in the database but which had significant homology to the catalytic domain of protein serine/threonine kinases. This fragment was used to

- 12 -

screen 500,000 plaques from a human B cell cDNA library in  $\lambda$ YES (provided by Stephen J. Elledge, Department of Biochemistry, Baylor College of Medicine). Seven positive clones were isolated, and those containing the largest inserts were analyzed by DNA sequencing of both strands using the dideoxy chain termination method with Sequenase 2.0 (USB, Inc.). DNA and amino acid sequence comparisons were made using the University of Wisconsin Genetics Computer Group programs BLAST, Pileup, and Bestfit, and the BEAUTY (BLAST Enhanced Alignment Utility) and BLASTPAT (BLAST PATtern database search tool) programs from the Human Genome Center, Baylor College of Medicine.

#### Northern blot analysis

Total RNA was isolated from rat organs by the guanidinium thiocyanate-phenol-chloroform method (Witzgall et al., *Mol. Cell. Biol.* 13:1933 (1993)). Twenty  $\mu$ g of total RNA was size-fractionated on a 1% formaldehyde-agarose gel and transferred to GeneScreen Plus (NEN) membrane as described. Id. Blots were hybridized with a 409-bp HindIII-BamHI fragment from the 3' half of SOK-1 (nucleotides 995-1403) which included 284 bp of open reading frame encoding part of the non-catalytic region, and 125 bp from the 3' untranslated region. This probe was labelled with [ $\alpha$ ]- $^{32}$ P dCTP by random priming. Hybridization was carried out for 18 hours at 45°C in 5X SSPE (1X SSPE: 150 mM NaCl, 10 mM  $\text{NaH}_2\text{PO}_4$ , 0.7 mM EDTA), 44% formamide, 5X Denhardt's solution, 1% SDS, 10% Dextran Sulfate, and 100  $\mu$ g/ml denatured salmon sperm DNA. The membranes were washed twice for 15 minutes at room temperature in 2X SSPE, twice for 30 minutes at 65°C in 2X SSPE with 2% SDS, and once for 30 minutes at room temperature in 0.2X SSPE.

- 13 -

Membranes were exposed to X-ray film for 5 days at -70°C with intensifying screens.

### Plasmids

Plasmids used included pMT3 (pMT2 modified to  
5 include sequence encoding the 9 amino acid hemagglutinin (HA) epitope N-terminal to the insert) (Pombo et al., Nature 377:750 (1995)), pCMV5 (a CMV-based vector including sequence encoding the 9 amino acid M2 epitope tag N-terminal to the insert), pEBG (a vector that is  
10 driven by the human EF-1 $\alpha$  promoter and that includes sequence encoding glutathione S-transferase (GST) N-terminal to the insert) (Pombo et al., Nature 377:750 (1995); Sanchez et al., Nature 372:794 (1994)), and pGEX-KG (a prokaryotic expression vector that includes  
15 sequence encoding GST N-terminal to the insert) (Guan et al., Anal. Biochem. 192:262 (1991)).

To create pCMV5-SOK-1 $\Delta$ C, pCMV5-SOK-1 was cut with HindIII and then religated. The pCMV5-SOK-1 $\Delta$ C construct contains sequence encoding amino acids 1-333 and includes  
20 the entire kinase domain of SOK-1, but not the carboxy terminal 93 amino acids of the protein. pEBG-SAPKp54 $\beta$ , pEBG-p38, pEBG-ERK1 contain the three MAP kinases p54 $\beta$  (the  $\beta$  isoform of the SAPK, p54), p38, and ERK1, respectively, as GST fusion proteins. pRSV-BXB-Raf-1  
25 encodes a variant of c-Raf-1 lacking the regulatory domain. BXB-Raf-1 is constitutively active and transforming (Bruder et al., Genes & Development 6:545 (1992); Pombo et al., Nature 377:750 (1995); Sanchez et al., Nature 372:794 (1994)).

### 30 Transfection protocols and kinase assays

Subconfluent COS7 cells were transfected using the DEAE-dextran technique as described (Pombo et al., Nature 377:750 (1995)). One to ten  $\mu$ g of expression plasmid DNA

- 14 -

were used per plate and adjusted to a total of 20 µg of DNA with the appropriate empty vector. Forty-eight hours after transfection, cells were exposed to various stimuli or vehicle, and extracts were prepared as described (Pombo et al., *Nature* 377:750 (1995); Pombo et al., *J. Biol. Chem.* 269:26546 (1994)). Extracts were exposed to anti-HA or anti-M2 (Kodak) monoclonal antibodies, or to an anti-SOK-1 rabbit polyclonal antibody (see below) for 3 hours, and immune complexes were collected with Protein G-Sepharose beads. Beads were washed three times in lysis buffer, three times in LiCl buffer (500 mM LiCl, 100 mM Tris-HCl, pH 7.6), and three times in assay buffer (Pombo et al., *J. Biol. Chem.* 269:26546 (1994)). Kinase assays were started by the addition of myelin basic protein (MBP, for SOK-1 and ERK1); GST-c-Jun (1-135), containing the transactivation domain of c-Jun (for SAPK) (Kyriakis et al., *Nature* 369:156 (1994); Pombo et al., *Nature* 377:750 (1995); Pombo et al., *J. Biol. Chem.* 269:26546 (1994)); or ATF-2 (8-94), containing the transactivation domain of ATF-2 (for p38) (Morooka et al., *J. Biol. Chem.* 270:30084 (1995)), in the presence of [ $\gamma$ ]-<sup>32</sup>P-ATP (100 µM, 3000-9000 cpm/pmole) and MgCl<sub>2</sub> (10 mM). After 5 to 20 minutes at 30°C, the kinase reactions were stopped with Laemmli sample buffer. Following SDS-polyacrylamide gel electrophoresis and autoradiography, the bands corresponding to the substrate were cut out of the gel and radioactivity was determined by liquid scintillation counting. For all kinase assays, an aliquot of the cell lysate was run on an SDS-polyacrylamide gel, transferred to Imobilon, and subjected to immunoblotting with the appropriate antibody to ensure equivalent expression of the kinases (Morooka et al., *J. Biol. Chem.* 270:30084 (1995)). Antibody binding was detected using the Enhanced Chemiluminescence System.



- 15 -

Phosphatase inactivation and reactivation experiments

Six 10 cm dishes of COS7 cells were transfected with pMT3-SOK-1. Forty eight hours later, cell lysates  
5 were subjected to immunoprecipitation with anti-HA antibody. Immune complexes were divided into equal aliquots and then incubated for 20 minutes at 30°C with the catalytic subunit of protein phosphatase 2A (PP2A), either with or without the PP2A inhibitor, okadaic acid  
10 (100 nM). PP2A was purified from rabbit skeletal muscle (Chen et al., *Science* 257:1261 (1992)) and was generously provided by Dr. David Brautigan (Center for Cell Signalling, University of Virginia Health Science Center). After the 20 minute incubation, okadaic acid  
15 was added to bring the final concentration to 100 nM in all tubes. Immune complexes were washed twice with kinase assay buffer, and then exposed to  $[\gamma]$ -<sup>32</sup>P-ATP (100 μM) for 0, 5, 10, or 20 minutes prior to the addition of MBP and subsequent kinase assay for 5 minutes at 30°C.

20 Production of anti-SOK-1 polyclonal antibodies

A peptide (amino acids 333-426) from the non-catalytic region of SOK-1 was used to generate a polyclonal rabbit antibody. This peptide was expressed in bacteria from pGEX-KG as a GST fusion protein,  
25 purified, and used to immunize rabbits according to standard protocols (Harlow et al., Antibodies: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1988)). The antibodies from each of two rabbits recognized 1 ng of GST-SOK1 on a  
30 Western blot when used at a 1:1000 dilution. In addition, at a 1:250 dilution, the antibody immunoprecipitated HA-SOK-1 from lysates of transfected cells.

- 16 -

Production of anti-SOK-1 monoclonal antibodies

Monoclonal antibodies can be generated using the standard Kohler and Milstein technique.

Microinjection and immunofluorescence

5        Mouse fibroblast NIH 3T3 cells were grown on glass coverslips and microinjected with the pMT3-SOK-1 expression vector, encoding SOK-1 with the HA epitope tag at its amino terminus. Plasmid DNA was purified twice on a CsCl gradient and extracted three times with phenol and  
10 chloroform. Cells were injected in a 3.5 cm dish with an automated microinjection system (AIS; Zeiss (Ansorge et al., *J. Biochem Biophys. Meth.* 16:283 (1988)) at a pressure between 80 and 170 kPa. The computer settings were as follows: angle, 45°; speed, 10; and time, 0.0  
15 sec. Plasmid DNA was injected at a concentration of 100 µg/ml concentration (Pagano, *Genes & Development* 8:1627 (1994)). Twenty-four hours after injection, the cells were fixed with 4% paraformaldehyde for 15 minutes, treated with 0.1% SDS in phosphate buffered saline (PBS)  
20 for 5 minutes, and permeabilized with 0.5% Triton X100 (in PBS) for 15 minutes. Cells were then processed for immunofluorescence (Brown et al., *J. Histochem. Cell. Biol.*, in press, 1996).

      All antibodies were diluted in Dulbecco's modified  
25 Eagle's medium containing 10% calf serum. Coverslips were incubated with affinity purified anti-HA antibody (Boehringer Mannheim) at a final concentration of 0.026 mg/ml for one hour. After incubation for 40 minutes in biotinylated goat anti-mouse antibody (Jackson  
30 Laboratories) which was diluted 1:50, the coverslips were incubated for 40 minutes in fluorescein isothiocyanate conjugated streptavidin, diluted 1:100 (Jackson Laboratories). All incubations were carried out at 37°C in a humidified chamber. Between each step, cells were

- 17 -

washed three times with PBS. Nuclei were counterstained with bisbenzimidazole (Hoescht 33258) for 2 minutes at 1 mg/ml in PBS. Coverslips were mounted in Crystal/Mount (Biomedica) and visualized on a Zeiss Axiovert 100 photomicroscope. Cells were imaged with a Bio-Rad Laser Scanning Confocal Microscope.

#### Derivation of SOK-1 kinase inactive mutants

A kinase inactive mutant of SOK-1 was derived by mutating the ATP binding site of SOK-1, by replacing the invariant lysine with arginine.

#### Identification and characterization of SOK-1, a novel Ste20 homolog

Screening of the human B cell cDNA library identified two clones of 1.8 kb and 2.0 kb. The nucleotide sequence of the gene encoded by these overlapping clones, and the deduced amino acid sequence of the protein encoded by this gene, are shown in Fig. 1. For the nucleotide sequence, numbers refer to the position of the codon relative to the initiator ATG. The predicted translation product is indicated below the nucleotide sequence, and the numbers refer to the position of the amino acid relative to the initial methionine. The 2.0 kb clone contains a Kozak sequence (gcggccatgg) at a candidate initiation codon (Fig. 1). There is an in-frame stop codon 15 bp 5' of this initiation codon. There are no other candidate initiation codons between this stop codon and the ATG, which suggests that this codon is the true translation start site. A poly (A) tail is present at the 3' end of the 1.8 kb clones.

The open reading frame encodes a protein which is 426 amino acids in length and has a predicted molecular mass of 48,041 daltons. The kinase domain is located in

- 18 -

the amino terminal half of the protein and contains all 11 subdomains of serine/threonine kinases (Hanks et al., *In Methods in Enzymology*, Hunter et al., eds., Academic Press, San Diego, CA, pp. 38-62 (1991)). Alignment of the catalytic domain of SOK-1 with the catalytic domains of the five most closely related kinases as determined by the BLAST and Bestfit programs is shown in Fig. 2. The deduced amino acid sequences of PAK1 (Manser et al., *Nature* 367:40 (1994)); Ste20 (Leberer et al., *EMBO J.* 11:4815 (1993)); MST1 (Creasy et al., *J. Biol. Chem.* 270:21695 (1995)); Sps1 (Friesen et al., *Genes & Development* 8:2162 (1994)); and GC kinase (Katz et al., *J. Biol. Chem.* 269:16802 (1994)) were aligned by eye after being aligned with the Pileup program. Gaps, which were introduced to maintain alignment, are denoted by dots. Roman numerals indicate the eleven protein serine/threonine kinase subdomains (Hanks et al., *In Methods of Enzymology*, Hunter et al., eds., pp. 38-62, Academic Press, Inc., San Diego, CA (1991)). Residues that are conserved in all family members are enclosed in boxes. Comparison of the amino acid sequence of the catalytic domain with other protein kinases using the BLAST program identified the yeast kinase, Sps1 (Friesen et al., *Genes & Development* 8:2162 (1994)), and the mammalian kinases, MST1 (Creasy et al., *J. Biol. Chem.* 270:21695 (1995)) and GC kinase (Katz et al., *J. Biol. Chem.* 269:16802 (1994)) as its closest homologs. Within the catalytic domain, SOK-1 was 50% identical and 68% similar to Sps1, 56% identical and 73% similar to MST1, and 51% identical and 68% similar to GC kinase.

The five kinases most closely related to SOK-1 are Sps1, MST1, GC kinase, Ste20 (Leberer et al., *EMBO J.* 11:4815 (1993)), and PAK1 (Manser et al., *Nature* 367:40 (1994)), all of which are Ste20 homologs. Alignment of the amino acid sequence of the catalytic domains of Sps1

- 19 -

and Ste20 with SOK-1 indicates a high degree of evolutionary conservation (Fig. 2). Comparison of the amino acid sequence of the C-terminal non-catalytic region of SOK-1 with the database using the BLAST, BEAUTY, and BLASTPAT programs failed to identify regions of significant homology with any other kinases.

SOK-1 is thus related to the Sps1 family of Ste20s on the basis of its organization, i.e., amino terminal catalytic domain, and sequence similarity within the kinase domain to the Sps1 group. SOK-1 is more similar in sequence to Sps1 (50% identical) than it is to Ste20 (42% identical). Furthermore, Sps1 is more similar to SOK-1 than Sps1 is to Ste20 (44% identical).

#### SOK-1 expression

mRNA was extracted from various rat tissues and subjected to Northern blot analysis using a probe from the carboxy terminal non-catalytic region of SOK-1. Expression of a 2300 bp mRNA was detected in all tissues examined except stomach, where the probe hybridized to two transcripts, one of approximately 2600 bp and one of 1500 bp (Fig. 3). Highest levels of expression were in testis, large intestine, brain, and stomach. Intermediate levels of expression were seen in heart and lung. Equal loading of all lanes in the gel was confirmed by ethidium bromide staining. The kinase was expressed in the two human B cell lines examined, Ramos, a Burkitt lymphoma cell line that has features of a germinal center B cell, and HS Sultan, a mature B cell line.

COS7 cells were transfected with PMT3-SOK-1, which encodes a SOK-1 protein with a nine amino acid HA epitope tag on the amino terminus. The results of Western blotting using a monoclonal anti-HA antibody as a probe of the PMT-3-SOK-1 transfected cells (+), as well as

- 20 -

cells transfected with the vector without the SOK-1 insert (-) are shown in Fig. 4A. Transfection with pMT3-SOK-1, but not the vector alone, resulted in expression of a protein with an approximate molecular weight of 50 kDa (indicated by the arrow in Fig. 4A). The kinase displayed a high degree of constitutive activity toward MBP in immune complex kinase assays. The results of a typical experiment in which COS7 cells were transfected with pMT3 vector alone (-), or 1  $\mu$ g (1) or 5  $\mu$ g (5) pMT3-SOK-1 is shown in Fig. 4B. Forty eight hours after transfection, the cells were harvested and lysates were subjected to immunoprecipitation with anti-HA antibody followed by immune complex assay using MBP as substrate. Phosphoamino acid analysis demonstrated that the kinase phosphorylated MBP on serine and threonine residues, but not on tyrosine.

To determine subcellular localization of SOK-1, pMT3-SOK-1, encoding HA-SOK-1, was microinjected into NIH3T3 fibroblasts at a concentration of 100  $\mu$ g/ml. HA-SOK-1 was detected by staining with the anti-HA antibody as described *supra*. SOK-1 was localized almost exclusively in the cytoplasm (Figs. 5B and 5E). A representative slice of 0.2  $\mu$ m of the same cells is also shown in Figs. 5A and 5D. The cells were counterstained with Hoescht 33258 to visualize the nuclei (Figs. 5C and 5F).

#### Regulation of SOK-1

The role of phosphorylation in the regulation of SOK-1 was explored. Exposure of SOK-1 to protein serine phosphatase 2A (PP2A) in immune complexes reduced SOK-1 kinase activity by approximately 40%. This effect of PP2A was prevented by co-incubation with the PP2A inhibitor okadaic acid (OA). To determine whether autophosphorylation might play a role in activating

- 21 -

SOK-1, the protein was partially inactivated by PP2A, then incubated with  $\gamma$ -<sup>32</sup>P-ATP (100  $\mu$ M) and assayed for reactivation (Fig. 6). Reactivation of SOK-1 kinase activity which correlated with phosphorylation of a 50 kDa protein in the immune complex. The increase in kinase activity over time correlated with the degree of phosphorylation. The phosphorylated protein also demonstrated enhanced electrophoretic mobility after PP2A treatment and retarded mobility after incubation with ATP (Fig. 6). The data thus suggest that phosphorylation, probably autophosphorylation, is an important mechanism of activation of SOK-1.

Autophosphorylation and autoactivation of a kinase in immune complexes, if unrecognized, greatly complicates the identification of activators. After a twenty minute incubation in the presence of ATP, MBP kinase activity of SOK-1 previously inactivated by PP2A, was equal to that of SOK-1 which had not been inactivated by PP2A (Fig. 6). Autophosphorylation and autoactivation may explain the difficulty which has been encountered in identifying activators of the Sps1 family of Ste20 homologs when standard immune complex kinase assays are performed. Under these conditions, no activators of MST1 were identified (Creasy et al., *J. Biol. Chem.* 270:21695 (1995)), and for GC kinase, TNF $\alpha$  only weakly stimulated kinase activity. Since SOK-1 is markedly activated by autophosphorylation in immune complex kinase assays, incubations for kinase assays of longer than 5 minutes can be expected to mask any differences between control and stimulated cells. Consequently, all subsequent kinase assays were performed for 5 minutes.

SOK-1 has an amino terminal catalytic domain, placing it, on the basis of organization, in the Sps1 group of Ste20s, which includes Sps1, GC kinase, and MST1. These kinases lack the Rac1/cdc42Hs binding domain

- 22 -

present in the regulatory domains of Ste20 and the PAK family of kinases, and the role of their carboxy terminal non-catalytic regions is unclear. The ability of the carboxy terminal region of SOK-1 to regulate kinase activity in transfected COS7 cells was tested (Fig. 7). Using MBP as a substrate, the kinase activity of SOK-1, expressed from pCMV5-SOK-1, which encodes SOK-1 with a nine amino acid M2 epitope tag at the amino terminus, was compared with that of M2-SOK-1ΔC, a deletion mutant containing the catalytic domain but missing the carboxy terminal 95 amino acids of the non-catalytic region, and pCMV5, which is the vector containing the M2 epitope tag, but lacking the SOK-1 sequences. Although the cellular extracts were matched for total protein prior to immunoprecipitation with anti-M2 antibody, immunoblots of the extracts revealed that M2-SOK-1ΔC was expressed at a much lower level than full-length M2-SOK-1 (Fig. 7, bottom). Despite the lower expression of M2-SOK-1ΔC, and the presence of much less M2-SOK-1ΔC compared to full-length M2-SOK-1 in the immunoprecipitates, kinase activity, measured as phosphorylation of MBP, was equivalent, consistent with significantly greater specific activity of M2-SOK-1ΔC (Fig. 7). These data suggest that the carboxy terminal non-catalytic region inhibits kinase activity of SOK-1 and is the first demonstration of a role for the non-catalytic region of protein kinases related to the Sps1 group of Ste20s. Inhibition of activity may be due to binding of the carboxy terminal region to the catalytic domain, since the carboxy terminal region (lacking the kinase domain) co-immunoprecipitates with SOK-1ΔC when the two are co-expressed. The carboxy terminal region may exert its inhibitory effect by preventing access by an activator, possibly SOK-1 itself, to a critical site within the



- 23 -

catalytic domain, or by inhibiting interaction of the kinase domain with substrates.

SOK-1 is thus regulated by its non-catalytic region, and by phosphorylation. Identification of these two regulatory mechanisms suggests that the regulation of SOK-1 may be similar to the regulation of PAK1. Binding of the inhibitory regulatory region of PAK1 to the small GTP binding proteins appears to allow the kinase to undergo autophosphorylation, which activates the kinase. For SOK-1, binding of the inhibitory regulatory region to an as yet unidentified activator may also allow autophosphorylation and activation to occur. Thus, the primary mechanism of activation of PAK1 and SOK-1 (and possibly other Ste20s) would be similar (autophosphorylation), but the activators to which the regulatory domains bind, allowing autophosphorylation to occur, would differ. Specificity in the activation of Ste20s (and subsequently, MAP kinase cascades) in response to a stimulus could be determined by protein or lipid interaction domains within the regulatory region.

#### Activation of SOK-1 by depletion of ATP stores

SOK-1 is markedly activated by the depletion of intracellular ATP stores, an important component of ischemia. Ischemia is a major cause of morbidity and mortality, and clinically presents as myocardial infarction, stroke, and acute renal failure. Several kinases are activated after reperfusion or after repletion of ATP stores, but SOK-1 is activated during the phase of ATP depletion, suggesting that it is a very early modulator of the response to ATP depletion and therefore ischemia.

#### Activation of SOK-1 by H<sub>2</sub>O<sub>2</sub>

Incubation of Ramos B cells with okadaic acid (1  $\mu$ M, 20 minutes) activated SOK-1 (Table 1), compatible

- 24 -

with regulation of SOK-1 (and/or an upstream activator) by phosphorylation. Numerous agonists that were representative of multiple different classes of stimuli were also tested for their ability to activate SOK-1.

5 Only  $H_2O_2$  consistently activated SOK-1 when native kinase was assayed after immunoprecipitation from Ramos B cells or when HA-tagged SOK-1 was assayed after immunoprecipitation from transfected COS7 cells (Table 1).  $H_2O_2$  (0.5 mM) activated SOK-1 approximately 3-fold (p  
10 < 0.01). No  $H_2O_2$ -induced increase in MBP kinase activity was detected when immunoprecipitation was performed with preimmune serum. Activation of SOK-1 was evident as early as 10 minutes following exposure of Ramos B cells to  $H_2O_2$ , peaked at 20 minutes, and remained elevated at 60  
15 minutes (Fig. 8). Activation was evident at 0.1 mM, the lowest concentration tested (2.1-fold increase in kinase activity). SOK-1 is thus markedly activated by oxidant stress. Oxidant stress is a prominent component of ischemia, and of reperfusion of ischemic tissue. Oxidant  
20 stress also occurs with ionizing radiation, such as ultraviolet or gamma radiation, and is an important element of inflammation.

This is the first clear demonstration of activation of a member of this group of Ste20s by any  
25 stimulus. The activation of SOK-1 by  $H_2O_2$  not only identifies a new oxidant stress response signal transduction pathway, but also suggests that one role of this and possibly other Ste20s of this group is to respond to environmental stresses just as their homologs  
30 do in the simplest eukaryotes. The survival of aerobic organisms depends upon their mounting an effective response to oxidant stress. Activation by oxidant stress suggests that SOK-1, and possibly other as yet unidentified SOK-1 homologs, may, like the Ste20s

- 25 -

identified thus far in yeast, play an important role in the responses of the cell to environmental stress.

In contrast to activation of SOK-1 by  $H_2O_2$ , potent activators of the ERK1/-2 cascade, such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF) and the phorbol ester phorbol myristate acetate (PMA) combined with the calcium ionophore, ionomycin, did not activate SOK-1 (Table 1). In the same COS7 cells, these agonists activated ERK1, expressed in pEBG, 5- to 7-fold. Oxidant stress appeared to be a specific activator among the several cellular stresses tested. Specifically, high and low osmolar stress, heat shock, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), and anisomycin, which potentially activate the SAPK and/or p38 cascades in these and other cells (see Figs. and Galcheva-Gargova et al., *Science* 265:806 (1994); Han et al., *Science* 265:808 (1994); Kyriakis et al., *Nature* 369:156 (1994); Pombo et al., *Nature* 377:750 (1995); Rouse et al., *Cell* 78:1027 (1994)), did not activate SOK-1. Platelet activating factor, which signals via a heterotrimeric G protein-coupled receptor and is a potent activator of intracellular  $Ca^{2+}$  transients in Ramos cells, was also ineffective.

- 26 -

Table 1. Fold-activation of native SOK-1 in Ramos B cells and HA-SOK-1 in COS7 cells.

<u>Agonist</u>	<u>Ramos</u>	<u>COS7</u>
H <sub>2</sub> O <sub>2</sub> (0.5 mM, 20 min)	2.9	2.8
5 Okadaic acid (1 $\mu$ M, 30 min)	2.3	-
Interferon- $\gamma$ (50 ng/ml, 20 min)	1.0	-
TNF $\alpha$ (50 ng/ml, 20 min)	1.5	0.9
Anti-Ig (20 min)	0.8	-
Platelet activating factor (1 $\mu$ M, 20 min)	1.4	-
10 PMA/Ionomycin (300 nM/1 $\mu$ M, 20 min)	1.4	1.4
Nitrogen mustard (10 $\mu$ M, 30 min)	1.2	1.4
Cyclophosphamide (10 $\mu$ M, 30 min)	0.9	1.5
cisplatin (10 $\mu$ M, 30 min)	1.0	1.1
Heat shock (42°C, 5 min)	-	0.9
15 Anisomycin (50 $\mu$ g/ml, 20 min)	-	1.1
Hyperosmolarity (NaCl 700 mM, 15 min)	-	0.9
Hypoosmolarity (150 mOsm, 15 min)	-	0.9
EGF (100 ng/ml, 10 min)	-	1.3
PDGF (20 ng/ml, 10 min)	1.0	1.2

20 - = not determined.

Native SOK-1 in Ramos B cells was assayed with MBP as substrate after immunoprecipitation with rabbit polyclonal anti-SOK-1. HA-SOK-1 was assayed after immunoprecipitation with anti-HA antibody from extracts  
 25 of COS7 cells which had been transfected with pMT3-SOK-1 (5  $\mu$ g per 10 cm dish). Hypoosmolar stress was induced by placing cells in Krebs-Henseleit buffer without NaCl (Pombo et al., 1994).

Reactive oxygen radicals, via damage to many  
 30 cellular components including DNA, can cause cell death, or if less severe, cell cycle arrest at either the G<sub>1</sub> or G<sub>2</sub> checkpoint (Russo et al., *J. Biol. Chem.* 270:29386

- 27 -

(1995)). DNA damage not only activates checkpoint controls, but may also activate protein kinases, including the SAPKs, c-Raf-1, and ERKs, which are integral components of cytoplasmic signal transduction cascades, as well as the non-receptor tyrosine kinase c-abl (Hibi et al., *Genes & Development* 7:2135 (1993); Kharbanda et al., *Nature* 376:785 (1995); Kharbanda et al., *J. Biol. Chem.* 270:18871 (1995); Livingstone et al., *EMBO J.* 14:1785 (1995); Radler-Pohl et al., *EMBO J.* 12:1005 (1993); Russo et al., *J. Biol. Chem.* 270:29386 (1995); Van Dam et al., *EMBO J.* 14:1798 (1995)). In order to determine whether activation of SOK-1 was likely to be triggered by DNA damage or by oxidant stress acting via a DNA damage-independent mechanism, alkylating agents were tested for their ability to activate SOK-1. Alkylating agents activate the DNA damage-induced checkpoint controls and protein kinases, but do not produce oxidant stress.

Exposure of transfected COS7 cells to the alkylating agents cyclophosphamide, nitrogen mustard, and cisplatin did not activate SOK-1, suggesting that oxidant stress-induced activation of SOK-1 is not mediated by DNA damage response pathways. Thus, activation of SOK-1 by oxidant stress is not part of a generalized response to either cellular or genotoxic stress. Cross-linking surface IgM on Ramos B cells with anti-Ig antibody coupled to beads, which induces apoptosis in these cells, did not activate SOK-1 but did markedly enhance tyrosine phosphorylation of several proteins in these cells.

Although these data clearly place SOK-1 on an oxidant stress response pathway, SOK-1 does not appear to activate the known stress-activated MAP kinase pathways. It has recently been reported that SOK-1 (previously called UK-1; the name was changed to SOK-1 to reflect the fact that the kinase is activated by oxidant stress),

- 28 -

unlike the closely related GC kinase, did not activate the SAPKs in co-transfection experiments (Pombo et al., *Nature* 377:750 (1995)). Co-transfection of HA-SOK-1 with the other MAP kinases, p38 (Fig. 9A) and ERK1 (Fig. 9B), both expressed in pEBG, did not result in the activation of the MAP kinases. In the p38 experiments, COS7 cells were transfected with pEBG vector (p38-) or pEBG encoding p38 as a GST fusion protein (p38+), and either pMT3 vector (SOK-1-) or pMT3 encoding HA tagged SOK-1 (SOK-1+). To confirm that p38 could be activated, cells were exposed to NaCl (500 mM) for 10 minutes (^NaCl+). p38 kinase activity was assayed with ATF-2 (8-94) as substrate (Morooka et al., *J. Biol. Chem.* 270:30084 (1995)). p38 was markedly activated by exposure of cells to osmolar stress.

In the ERK1 experiments, COS7 cells were transfected with pEBG vector (ERK1-) or pEBG encoding ERK1 as a GST fusion protein (ERK1+), and either pMT3 vector (SOK-1-), pMT3 encoding HA-tagged SOK-1 (SOK-1+), or as a positive control, pMT3 encoding BXB-Raf (+), a constitutively active c-Raf-1 that is missing the amino terminal regulatory domain (Bruder et al., *Genes & Development* 6:545 (1992)). ERK1 assays were performed in duplicate with MBP as substrate. As shown in Fig. 9B, ERK1 was activated by co-transfection of pRSV-BXB-Raf-1, but not SOK-1.

Oxidant stress activates the ERKs, and may activate the SAPKs somewhat (Kyriakis et al., *Nature* 369:156 (1994); Russo et al., *J. Biol. Chem.* 270:29386 (1995)), but SOK-1 does not appear to be implicated in this activation. SOK-1 did not activate any of four MAP kinase cascades, including SAPKs (Pombo et al., *Nature* 377:750 (1995)); p38 (Fig. 9A); ERK1 (Fig. 9B); or MEK5/ERK5 (Zhou et al., *J. Biol. Chem.* 270:12665 (1995)), further indicating that the stress response pathway

- 29 -

regulated by SOK-1 is unique. Since evolutionary conservation of the activation of MEKK/MEK/MAPK cascades by Ste20s extends to mammals (Polverino et al., *J. Biol. Chem.* 270:26067 (1995)); Pombo et al., *Nature* 377:750 (1995); Zhang et al., *J. Biol. Chem.* 270:12665 (1995)), and all Ste20s identified to date in yeast or mammals, with the exception of MST1 (Creasy et al., *J. Biol. Chem.* 270:21695 (1995)) have been shown to activate one or more MAP kinase cascades, it is likely that SOK-1 controls a novel oxidant stress-activated MAP kinase cascade.

#### SOK-1 functions

NF $\kappa$ B is a ubiquitously expressed transcription factor that is believed to be critical to diverse processes including T lymphocyte activation, the expression of cellular adhesion molecules, and the expression of interferon  $\beta$ . NF $\kappa$ B appears to play vital roles in transplant rejection, post-ischemic injury, the response to viral infection, and inflammation. Many diverse genes are believed to be under the control of NF $\kappa$ B. NF $\kappa$ B is activated by cytokines, such as TNF $\alpha$ , IL-1 $\beta$ , and IL-2; lipopolysaccharide, the mediator of septic shock; viruses, including Human T Cell Leukemia Virus Type 1, Human Immunodeficiency Virus 1, and Hepatitis B; ultraviolet and X-irradiation; antigen stimulation of T and B lymphocyte receptors; and the tumor promoting phorbol esters. In addition, most, if not all, of the activators of NF $\kappa$ B result in oxidant stress. Therefore, SOK-1 could be a final common pathway for activation of NF $\kappa$ B, and SOK-1 having an inactive kinase domain could be a general inhibitor of NF $\kappa$ B. In order to test these hypotheses, reporter plasmids containing NF $\kappa$ B binding sites linked to the Interleukin 2 (IL-2) receptor  $\alpha$ -chain promoter were constructed. SOK-1 activated IL-2 receptor  $\alpha$ -chain expression from these constructs, indicating that

- 30 -

SOK-1 activates NF $\kappa$ B. In addition, transfection of SOK-1 causes an increase in the binding of a nuclear protein to an oligonucleotide containing a consensus NF $\kappa$ B binding site.

5           A kinase inactive mutant of SOK-1 was constructed by changing the invariant lysine in the ATP binding site to an arginine. This kinase inactive mutant suppresses nuclear protein binding to the oligonucleotide containing the NF $\kappa$ B consensus binding site. Transcription from the  
10 NF $\kappa$ B reporter plasmid is also inhibited by the mutant protein. The kinase inactive mutant thus serves as a dominant inhibitor of activation of NF $\kappa$ B. Inhibitors of NF $\kappa$ B have not heretofore been identified, although they have been sought extensively, since it is believed that  
15 inhibition or stimulation of NF $\kappa$ B in inflammatory and autoimmune diseases, as well as cancer or viral infection, may be palliative or curative. Verma et al., *Genes & Development* 9:2723 (1995).

#### SOK-1 and Cell Cycle Arrest

20           Experiments were performed to investigate the role of SOK-1 in the induction of cell cycle arrest, which occurs in many types of cells following oxidant stress. In these experiments, NIH3T3 cells on coverslips were synchronized in G<sub>0</sub> by serum withdrawal. After twenty-  
25 four hours, less than 1% of the cells continued to cycle. Arrested cells were released with 10% calf serum, and were microinjected in early G<sub>1</sub> phase with the pCMV5 vector alone, or pCMV5 containing the gene encoding M2 epitope-tagged SOK-1. Entry into S phase was determined  
30 by monitoring BrdU (0.1 mM) incorporation. After microinjection of the pCMV5 vector alone, over 90% of cells entered S phase. In contrast, after injection of pCMV5-SOK-1, less than 5% of cells entered S phase.



- 31 -

Injection of a kinase inactive mutant of SOK-1 also induced cell cycle arrest (<5% of cells in S phase).

Since both SOK-1 and the kinase inactive mutant were effective in maintaining cells in G<sub>1</sub>, the non-catalytic carboxy terminal region of the kinase might be mediating cell cycle arrest. To test this hypothesis, pCMV5 containing only the non-catalytic region (nucleotides 858 to 1278, encoding amino acids 286 to 426) of the SOK-1 gene was injected into NIH3T3 cells. Like the constructs containing the full length SOK-1 or the kinase inactive mutant, this construct also induced G<sub>1</sub> arrest (<5% of cells in S phase). SOK-1 thus potentially induces arrest in G<sub>1</sub> of the cell cycle, via a mechanism that is not dependent upon the protein's catalytic function. A fragment of the SOK-1 polypeptide of approximately forty amino acids, from amino acid 286 to 336, may be sufficient to induce cell cycle arrest.

SOK-1-mediated cell cycle arrest occurs independently of the p38 and other MAP kinases that are known to induce cell cycle arrest. The ability of SOK-1 to induce cell cycle arrest, as well as to activate NFκB, makes SOK-1 an ideal target for drug development. The ability of SOK-1 to cause cell cycle arrest also suggests that it could be used following balloon angioplasty-induced injury of blood vessels, in order to inhibit the proliferative response which accompanies such injuries and causes re-stenosis. SOK-1 can also be used to treat other conditions that are characterized by proliferative responses, including inflammatory responses, tumors, and conditions such as atherosclerosis.

### Transgenic Animals

SOK polypeptides can also be expressed in transgenic animals. SOK transgenic animals are useful for screening for compounds that enhance or down regulate SOK

- 32 -

expression or activity. Animals of any species, including, but not limited to, mice, rats, rabbits, guinea pigs, pigs, micro-pigs, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees, can be used to generate SOK expressing transgenic animals.

Various techniques known in the art can be used to introduce a SOK transgene into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., *Proc. Natl. Acad. Sci. USA*, 82:6148 (1985); gene targeting into embryonic stem cells (Thompson et al., *Cell*, 56:313 (1989)); and electroporation of embryos (Lo, *Mol. Cell Biol*, 3:1803 (1983)).

The present invention provides for transgenic animals that carry the SOK transgene in all their nucleated cells, as well as animals that carry the transgene in some, but not all of their nucleated cells, i.e., mosaic animals. The transgene can be integrated as a single transgene or in concatamers, e.g., head-to-head tandems or head-to-tail tandems. For example, transgenic animals can be made in which SOK-1 is under the control of an inducible promoter. The transgene can also be selectively introduced into and/or activated in a particular cell type. Lasko et al., *Proc. Natl. Acad. Sci. USA*, 89:6232 (1992). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. -1 gene, gene. Vectors containing some nucleotide sequences homologous to an endogenous SOK gene can be designed for the purpose of integrating via homologous recombination into the endogenous gene and disrupting its function, i.e., making "knockout mice." The transgene also can be

- 33 -

selectively introduced into a particular cell type, thus inactivating the endogenous SOK-1 gene in only that cell type. See Gu et al., *Science*, 265:103 (1984). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant SOK gene can be assayed utilizing standard techniques. Initial screening can be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to assay whether integration of the transgene has taken place. The level of mRNA expression of the transgene in the tissues of the transgenic animals can also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and RT-PCR. Samples of tissues expressing SOK can also be evaluated immunocytochemically using antibodies specific for the SOK transgene product.

#### Therapeutic compositions

The therapeutic compositions of the invention can be used to increase SOK expression or activity in a patient to treat a pathological condition, e.g., a condition associated with a proliferative response, such as inflammatory responses, cancer, atherosclerosis or balloon angioplasty-induced injury to blood vessels. The therapeutic compositions of the invention can also be used to treat pathological conditions associated with NKFB expression, such as transplant rejection, post ischemic injury, and the response to viral infection. These compositions can contain the polypeptides or DNAs of the invention, including SOK-1 or a fragment thereof,

- 34 -

or a kinase inactive mutant of SOK-1. Polypeptides can be purified by methods that are known to those skilled in the art. Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1995. DNAs can be administered in a manner allowing their uptake and expression by cells in vivo. DNAs can be administered to the patient by standard vectors and/or gene delivery systems. Suitable gene delivery systems include liposomes, biolistic transfer, receptor-mediated delivery systems, naked DNA and viral vectors such as herpes viruses, retroviruses, adenoviruses and adeno-associated viruses. The polypeptides and DNAs of the invention are administered with a pharmaceutically acceptable carrier, and are formulated according to procedures that are well known to those skilled in the art.

Parenteral administration, such as intravenous, subcutaneous, intramuscular or intraperitoneal delivery routes can be used to deliver the therapeutic compositions of the invention. Dosages for particular patients depend upon many factors, including the patient's size, body surface area, age, the particular substance to be administered, time and route of administration, general health and other drugs being administered concurrently. The amount of therapeutic composition to be administered to a patient can be in the range of 1 to 1000  $\mu\text{g/kg}$  of body weight, e.g., 10 to 500, or 20 to 200  $\mu\text{g/kg}$  of body weight. A typical dose of polypeptide or DNA to be administered to a patient is 100  $\mu\text{g}$  per kilogram of body weight.

### 30 Diagnostic applications

Anti-SOK-1 antibodies can be used to assay tissues for SOK-1; elevated SOK-1 levels may be indicative of cell stress caused, e.g., by ischemia resulting from insults such as stroke and myocardial infarction.

- 35 -

Immunoassays using anti-SOK-1 antibody are carried out by standard techniques; e.g., the antibody can be labelled with a detectable label and contacted with a tissue sample under conditions which allow immune complexes to form. The uncomplexed labelled antibody is removed, and labelled immune complexes measured as a measure of SOK-1 in the sample. Immunoassays that can be performed using SOK-1 antibodies are well known to those skilled in the art. See e.g., Ausubel et al., Current Protocol in  
10 Molecular Biology 2: 11:2, John Wiley & Sons, 1995. Immunoassays can utilize radioactive, enzyme-based, or chemiluminescent labels.

- 36 -

What is claimed is:

1. An isolated DNA having a nucleotide sequence encoding a SOK polypeptide, or a biologically active fragment thereof, or the complement of said sequence.
- 5        2. The isolated DNA of claim 1, said DNA encoding an amino acid sequence with at least 50% sequence identity to the amino acid sequence set forth in Fig. 1 (SEQ ID NO:2).
- 10       3. The isolated DNA of claim 1, wherein said DNA encodes the amino acid sequence set forth in Fig. 1 (SEQ ID NO:2).
4. A DNA at least 10 nucleotides in length which hybridizes to the nucleotide sequence of Fig. 1 (SEQ ID NO:1), under stringent conditions.
- 15       5. The isolated DNA of claim 1, wherein said nucleic acid is cDNA.
6. The isolated nucleic acid of claim 1, wherein said nucleic acid is genomic DNA.
7. A substantially pure preparation of a SOK  
20 polypeptide, or a biologically active fragment thereof.
8. The preparation of claim 7, wherein said SOK polypeptide is human.
9. A vector comprising the DNA of claim 1.
10. A cell containing the DNA of claim 1.

- 37 -

11. A method of determining whether a candidate compound modulates SOK expression or activity, said method comprising the steps of:

- a) providing a first and a second recombinant  
5 cell expressing said SOK gene;
- b) introducing a candidate compound into said first cell, but not into said second cell;
- c) measuring a SOK function in said first and second cells; and
- 10 d) comparing the results obtained with said first and second SOK-transformed cells, wherein an increase or decrease in said first cell compared to second cell is an indication that said candidate compound modulates SOK gene expression or activity.

15 12. The method of claim 11, wherein said function is activation of DNA encoding NFkB.

13. The method of claim 11, wherein said function is protein kinase activity.

20 14. The method of claim 11, wherein said function is arrest of the cell cycle.

15. The method of claim 11, wherein said function is mediated by the activation of an SOK polypeptide by  $H_2O_2$ .

25 16. A therapeutic composition comprising as an active ingredient the polypeptide of claim 7, said polypeptide being formulated in a pharmaceutically acceptable carrier.

- 38 -

17. A therapeutic composition comprising as an active ingredient the DNA of claim 1, said DNA being formulated in a pharmaceutically acceptable carrier.

18. A kinase-inactive mutant of a SOK  
5 polypeptide.

19. Isolated DNA encoding the mutant of claim 18.

20. A therapeutic composition comprising as an active ingredient the polypeptide of claim 18, said polypeptide being formulated in a pharmaceutically  
10 acceptable carrier.

21. A therapeutic composition comprising as an active ingredient the DNA of claim 19, said DNA being formulated in a pharmaceutically acceptable carrier.

22. A method of inhibiting in a mammal the  
15 activation of the gene encoding NF $\kappa$ B, said method comprising administering to said mammal an NF $\kappa$ B inhibiting amount of the therapeutic composition of claim 20.

23. The method of claim 22, wherein said NF $\kappa$ B  
20 inhibiting amount is 20 to 200  $\mu$ g/kg of body weight.

24. A method of treating a blood vessel in a mammal which has undergone balloon angioplasty, said method comprising administering a therapeutically effective amount of the composition of claim 16.

25. The method of claim 24, wherein said  
25 therapeutically effective amount is 20 to 200  $\mu$ g/kg of body weight.



- 39 -

26. A method of producing a SOK polypeptide, said method comprising the steps of:

culturing a cell comprising an isolated DNA encoding said SOK polypeptide under conditions permitting  
5 expression of said SOK polypeptide from said DNA, and isolating said expressed SOK polypeptide.

27. A substantially pure antibody which specifically binds SOK.

28. A kit comprising  
10 an anti-SOK antibody bound to a solid support, said antibody being capable of specifically forming an immunocomplex with SOK in a sample; and means to detect said immunocomplex.

29. A biologically active fragment of an SOK-1  
15 polypeptide.

30. The fragment of claim 29, wherein said fragment comprises the noncatalytic region of SOK-1.

31. Isolated DNA encoding the fragment of claim  
25.

1/6

caccga

ggccectggtgtccctcgtagtggactgacgccgcagggcgagctagccggctccgcgc  
 ctctccgcggatccagacgcctcctggggctgctggcgagggtctgaggcgccgcggcc  
 atggctcacctccggggatttgcaaaccagcactctcgsgtggacctgaggagctcttc 60  
 M A H L R G F A N Q H S R V D P E E L F 20  
 accaagctcgaccgcattggcaagggtcgtttggggaggtctacaagggtcatcgataac 120  
 T K L D R I G K G S F G E V Y K G I D N 40  
 cacacaaaggaggtggtggccatcaagatcatcgacctggaggaggccgaggatgagatc 180  
 H T K E V V A I K I I D L E E A E D E I 60  
 gaggacatccagcaggagatcactgtcctcagtcagtcgacagccctacatcacccgc 240  
 E D I Q Q E I T V L S Q C D S P Y I T R 80  
 tactttggctcctacctaagagcaccaagctatggatcatcatggagtacctggggcggc 300  
 Y F G S Y L K S T K L W I I M E Y L G G 100  
 ggctcagcactggacttgcttaaaccaggtcccctggaggagacatacattgccacgac 360  
 G S A L D L L K P G P L E E T Y I A T I 120  
 ctgcgggagattctgaagggtcctggattatctgcactccgaacgcaagatccaccgagac 420  
 L R E I L K G L D Y L H S E R K I H R D 140  
 atcaaagctgccaacgtgctactctcgagcaggggtgacgtgaagctggcggaactttggg 480  
 I K A A N V L L S E Q G D V K L A D F G 160  
 gtagcagggcagctcacagacacgcagattaagaggaacacattcgtgggcaccccccttc 540  
 V A G Q L T D T Q I K R N T F V G T P F 180  
 tggatggcacctgaggtcatcaagcagtcggcctacgacttcaaggctgacatctggtcc 600  
 W M A P E V I K Q S A Y D F K A D I W S 200  
 ctgggcatcacagccatcgagctggccaagggggagcctccaaactctgacctccacccc 660  
 L G I T A I E L A K G E P P N S D L H P 220  
 atgcgcgtcctgttcctgattcccaagaacagccccacacactggaggggccagcacagc 720  
 M R V L F L I P K N S P P T L E G Q H S 240  
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 K P F K E F V E A C L N K D P R F R P T 260  
 gccaaaggagctcctgaagcacaagttcatcacacgctacaccaagaagacctccttcctc 840  
 A K E L L K H K F I T R Y T K K T S F L 280  
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 T E L I D R Y K R W K S E G H G E E S S 300  
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 S E D S D I D G E A E D G E Q G P I W T 320  
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 F P P T I R P S P H S K L H K G T A L H 340  
 agttcacagaagcctgcccagcgcctcaagaggcagccgaggtcccagtgccctgtccacg 1080  
 S S Q K P A D A V K R Q P R S Q C L S T 360  
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 L V R P V F G E L K E K H K Q S G G S V 380  
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 S D K L M V H L V E R V Q R F S H N R N 420  
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 atgttttgtttttaagaaaattgaattacttgtttcctgaaaaaaaaa 1849

FIG. 1

2/6

	I		II		
PAK1	PKKKYTRFEK	IGOGASGTVY	TAMDVATGOE	VATKQMNLOQ	.OPKKELIIN
Ste20	PSTKYANLVK	IGOGASGGVY	TAYEIGQNV	VATKQMNLEK	.OPKKELIIN
MST1	PEEVFDVLEK	IGEGSYGSVY	KAIHKETGOI	VATKQVPV..	.ESDLQEIHK
SOK-1	PEELFTKLDR	IGKGSFGEVY	KGIDNHITKEV	VATKIIDLEE	AEDEIEDIQQ
Sps1	PSKLYSIQSC	IGFGNFQDVY	KAVDRTQOEI	VATKVVNLEH	SDEIDIELLAQ
GCK	HRDRFELLQR	VGAGTYGQVY	KARDTVQSEL	AVKIVKLD.	PGDDISSLQQ
	III		IV		V
PAK1	EILVMRENKN	PNIVNYLDSY	LVGDEIWMVM	EYLAGGSLTD	VV...TETCM
Ste20	EILVMKGSKH	PNIVNFIDSY	VLKGDIVWIM	EYMEGGSLTD	VV...THCIL
MST1	EISIMQOCDS	PHVVKYIGSY	FKNTDIWIM	EYCGAGSVSD	IIRLR.NKTL
SOK-1	EITVLSQCD	HYITRYFGSY	LKSTKIWIIM	EYLGGSALD	L..LK.PGPL
Sps1	EIEFLAELKS	ELITNYIATM	LEDVSMWIM	EYCGGGSQSD	LLKRSYVNG
GCK	EITILRECRH	ENVVAYIGSY	LRNDRIWIM	EFCGGGSLOE	IYH..ATGPL
	VI		VII		
PAK1	DEGOIAAVCR	ECLQATIEFLH	SNQVIHRDIK	SDNLLIGMDG	SVKIDDFGFC
Ste20	TEGOIGAVCR	ETLSGLEFLH	SKGVIHRDIK	SDNLLISMEG	DIKIDDFGFC
MST1	TEDEIATILQ	STLKGLEFLH	FMRKIHRDIK	AGNLLINTEG	HAKIADFGVA
SOK-1	EETIATILR	EILKGLDYHL	SERKIHRDIK	AANLLISEQG	DVKIADFGVA
Sps1	PEEKVSFIIH	EVTLGKLYHL	EORKIHRDIK	AANLLINEEG	MVKIADFGVS
GCK	ERQIAYVCR	ERLKGTHHLH	SOGKIHRDIK	GANLLITLQG	DVKIADFGVS
	VIII		IX		
PAK1	AQITPEOSKR	STMVGTFTWM	APEVVTTRK..	.AYGPKVDIWM	SLGIMATIMI
Ste20	AQINELNIKR	TTMVGTFTWM	APEVVSRRK..	.EYGPVKVDIWM	SLGIMIMIMI
MST1	GOLTDTMAKR	NTVIGTFTWM	APEVI..Q.E	IGYNCVADIWM	SLGITATIMA
SOK-1	GOLTDTOIKR	NTFVGTFTWM	APEVI..K.Q	SAYDFKADIWM	SLGITATIELA
Sps1	GHIRSTL.KR	DTFVGTFTWM	APEVVCCE.V	DGYNEKADIWM	SLGITITIELL
GCK	GELTASVAKH	RSFVGTFTWM	APEVAAVERK	GGYNELODIWM	ALGITATIELG
	X		XI		
PAK1	EGEPEYLNEN	ELRALYLIAT	NG..TPELQN	PEKLSAIFRD	ELNRCTEMDV
Ste20	EGEPEYLNEN	ELRALYLIAT	NG..TPKLKE	PENLSSSLKK	ELDWCLCVP
MST1	EGKPEYADIH	EMRAIFMIPT	NP..PFTFRK	PELWSDNFTD	EVKQCLVKSP
SOK-1	KGEPENSDLH	EMRVLFLLPK	NS..PFTLEG	QH..SKPFKE	EVEACINKDP
Sps1	KGLPELSKYD	EMKVMTNLPK	RK..PFRLOG	P..FSDAAKD	EVAGCLVKTP
GCK	ELQPELFHLH	EMRALMLMSK	SSFQPEKLRD	KTRWTQNFHH	ELKLALTKNP
PAK1	EMKGSARELI	QHOF.LKIAK	PLSSITPL..		
Ste20	EDRASATELI	HDEYITEIAE	ANSSIAPLVK		
MST1	EQRATATOLI	QHPFV.RSAK	GVSTIRDLIN		
SOK-1	RFRPTAKELI	KHKFITRYTK	KTSFTELID		
Sps1	ADRPSPAYNLI	SFEFVKNIT.	.ITNKSQVD		
GCK	KRPPTAEKLI	QHPFTTQQLP	R.AITQLLD		

FIG. 2

3/6

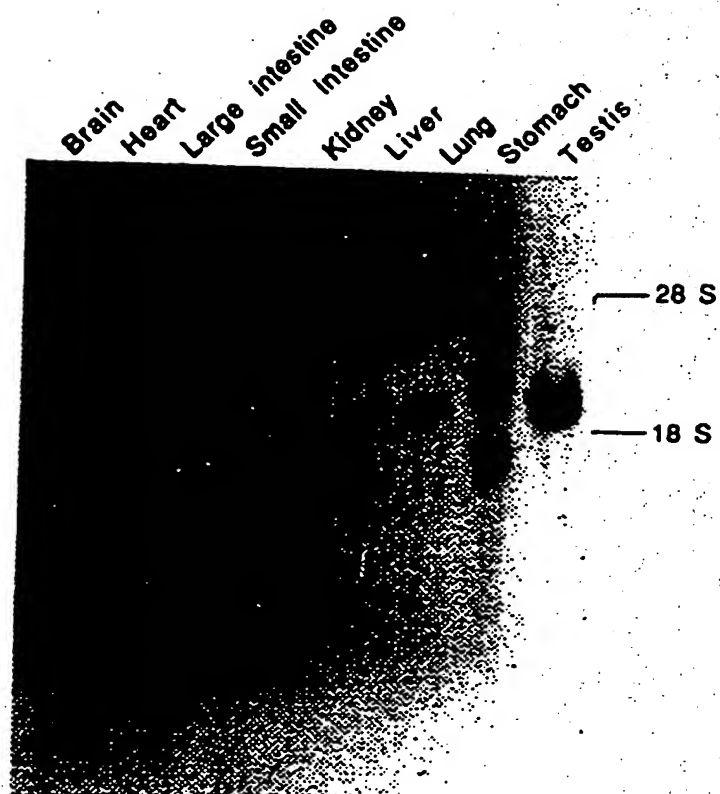


FIG. 3

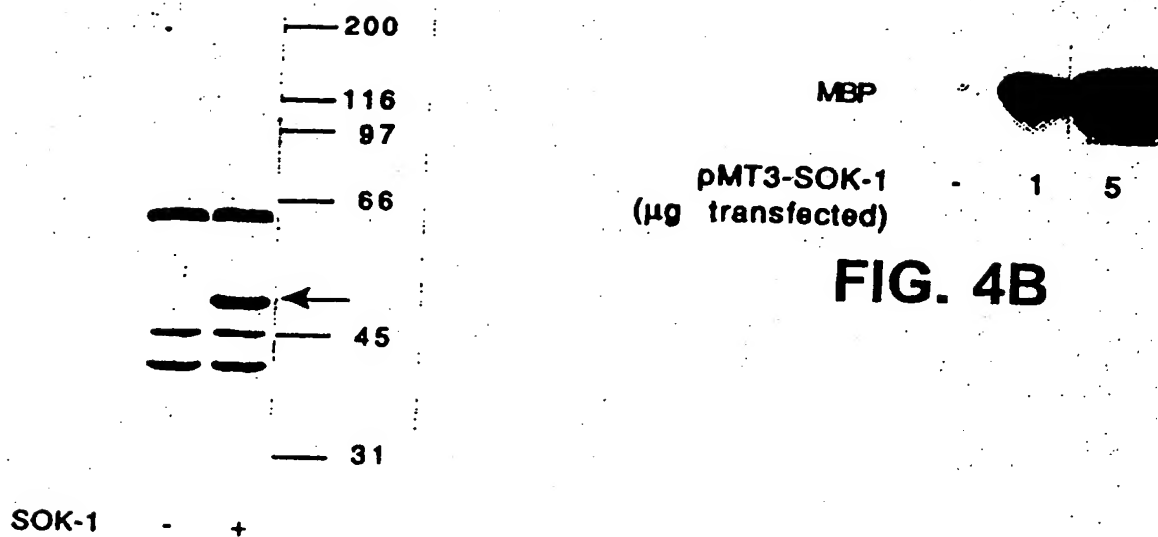


FIG. 4A

FIG. 4B

FIG. 5A



FIG. 5B



FIG. 5C



FIG. 5D

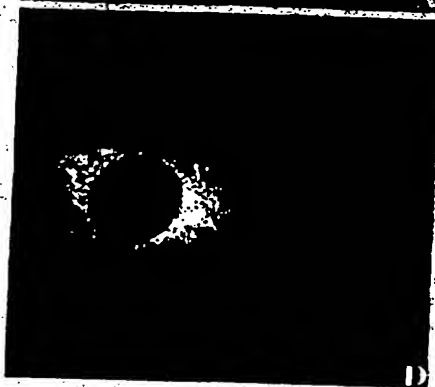


FIG. 5E



FIG. 5F

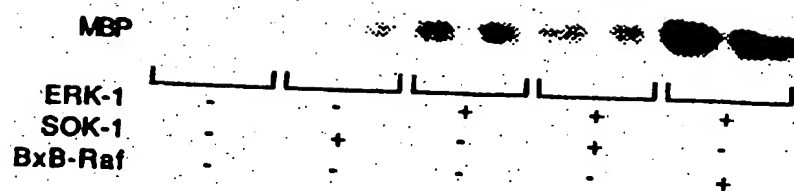
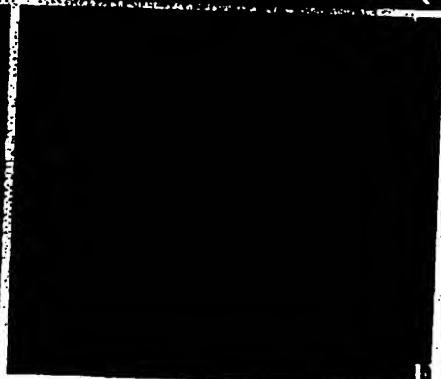


FIG. 9B

5/6

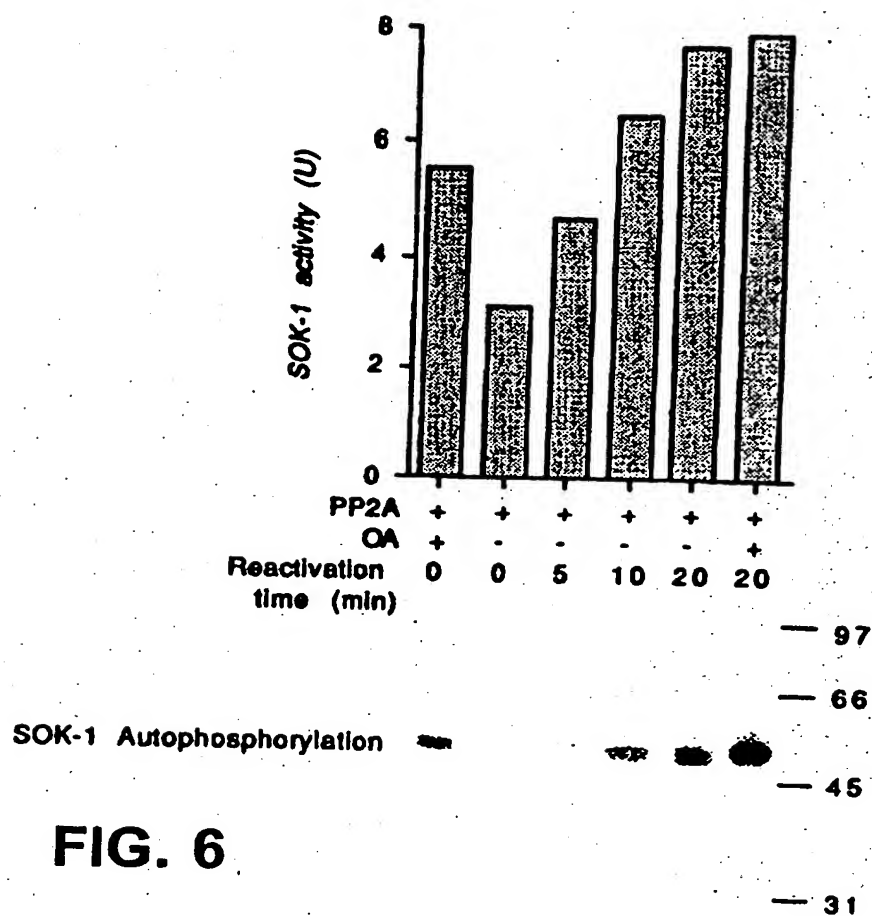


FIG. 6

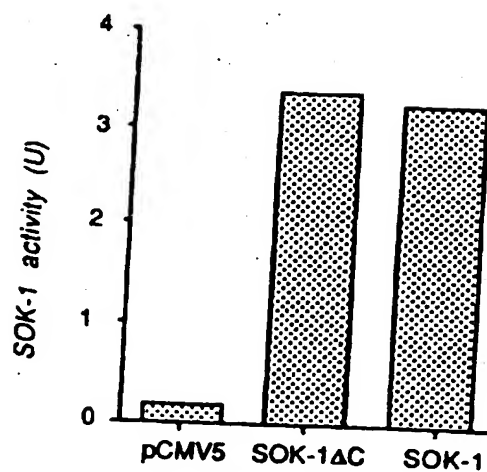
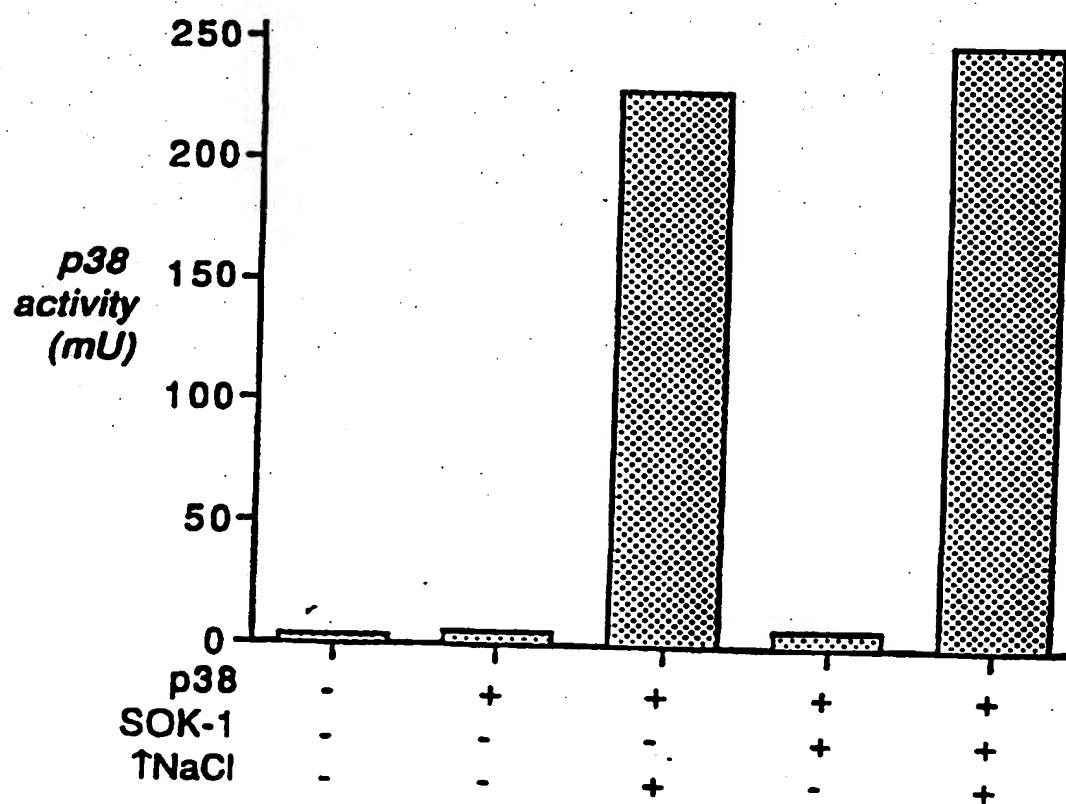
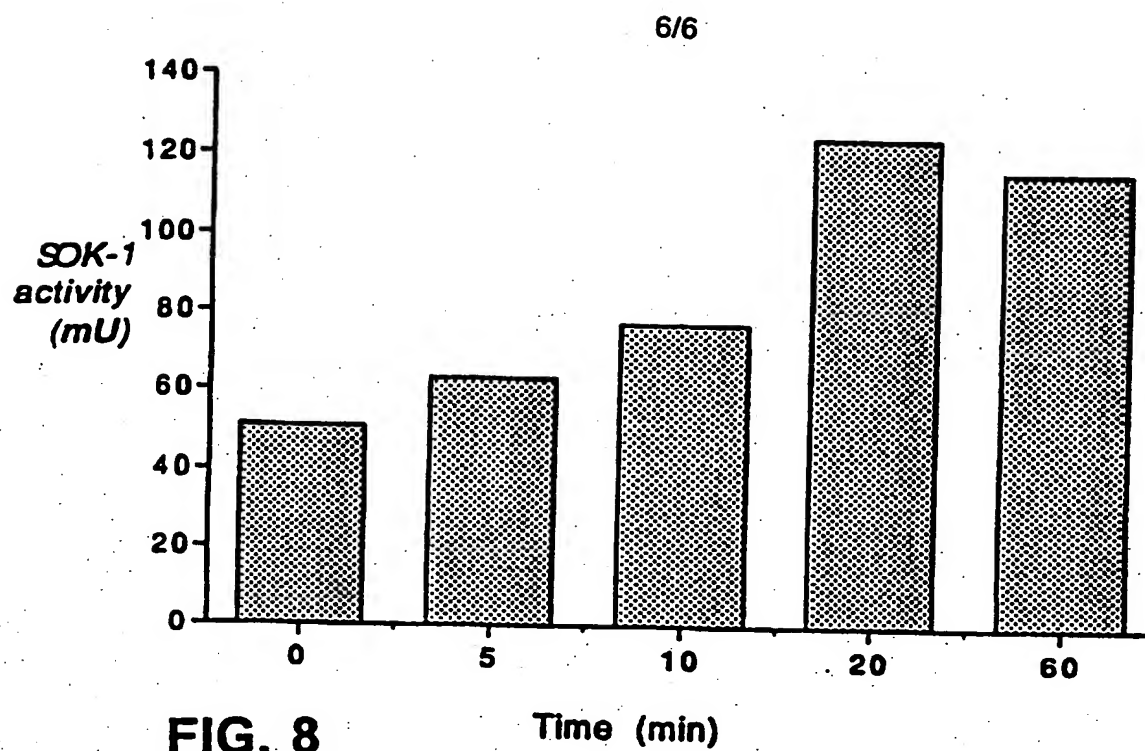


FIG. 7



**FIG. 9A**

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/07739

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07H 21/04; C12N 9/12, 15/00, 1/20, 5/00; A61K 38/51; A01N 43/04

US CL : 536/23.2; 435/194, 320.1, 252.3, 254.11, 325; 424/94.5; 514/44

According to International Patent Classification (IPC) or to both national classification and IPC.

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.2; 435/194, 320.1, 252.3, 254.11, 325; 424/94.5; 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS and STN (bioscience and patents indexes): mitogen activated protein kinase#, MAPK, human, rat, mammalian, Sterile 20, STE20, SPS1, Sporulation Specific, protein kinase#, oxidant, stress

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, P	OSADA et al. YSK1, a Novel Mammalian Protein Kinase Structurally Related to Ste20 and SPS1, But Is Not Involved in the Known MAPK Pathways. Oncogene. April 1997. Vol. 14. No. 17. pages 2047-2057, see entire document.	1-10, 16-23, 26, 29-31
A, E	POMBO et al. Activation of a Human Ste20-like Kinase by Oxidant Stress Defines a Novel Stress Response Pathway. EMBO Journal. September 1997. Vol. 15. No. 17. pages 4537-4546, see entire document.	1-10, 16-23, 26, 29-31

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

Special categories of cited documents:	
*A* document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*E* earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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*O* document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

19 AUGUST 1997

Date of mailing of the international search report

10 SEP 1997

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# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US97/07739

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:  
1-10, 16-23, 26, 29-31

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

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